Original Article

Molecular Identification and Genotyping of Babesia canis in Dogs from Meshkin Shahr County, Northwestern Iran

***Majid Khanmohammadi[1]; Reza Zolfaghari-Emameh[2]; Mehdi Arshadi[3]; Elham Razmjou[4]; Poorya Karimi[4]**

[1]Department of Laboratory Sciences, Marand Branch, Islamic Azad University, Marand, Iran
[2]Department of Energy and Environmental Biotechnology, Division of Industrial and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran
[3]Al-Zahra Hospital, Tabriz University of Medical Sciences, Tabriz, Iran
[4]Department of Medical Parasitology and Mycology, Iran University of Medical Sciences, Tehran, Iran

***Corresponding author:** Dr Majid Khanmohammadi, E-mail: Majid593@gmail.com

(Received 12 Oct 2019; accepted 13 Mar 2021)

Abstract

Background: Canine babesiosis is one of the mainly worldwide-distributed tick-borne haemoprotozoan parasitic diseases in dogs.

Methods: A total of 43 blood samples were randomly collected from naturally infected dogs in seven villages from different geographical areas of Meshkin Shahr, Ardabil Province, Iran. The presence of *Babesia* species detected with standard methods including parasitological and gene sequencing techniques targeting the 18S rRNA gene.

Results: Our results revealed that four dogs 9.3% (4/43) including one female and three male dogs were infected with *Babesia*. All four *Babesia*-infected dogs were confirmed *B. canis* by the molecular-based method. Sequence alignments comparison of the *B. canis* genotypes A and B, it was revealed that all *B. canis* isolates belonged to genotype B.

Conclusion: This study provides essential data for subsequently define the critical importance of the molecular studies in management and prevention of the canine babesiosis in Iran.

Keywords: *Babesia canis*; Babesiosis; Dogs; Genotyping; RNA, ribosomal, 18S; Iran

Introduction

Canine babesiosis is a tick-borne parasitic disease with worldwide importance and caused by intra-erythrocytic *Babesia* species. The identification of each *Babesia* species routinely is based on the host specificity and the morphological characteristics of piroplasmids (1). The differences in geographical distribution, vector specificity, antigenic properties, genetic characteristics and severity of the clinical manifestations sub divided the former species into the three subspecies, namely *B. canis canis* (3–5μm) is transmitted by *Dermacentor reticulatus* in Europe, *B. canis vogeli* transmitted by Rhipicephalus sanguineus sensu lato in tropical and subtropical regions, and *B. canis rossi* transmitted by *Haemaphysalis leachi* in South Africa (1, 2).

Babesia gibsoni (1.5–2.5μm) is present in Asia, North America, Africa, Australia and Europe (3-6). The geographical distributions of both species of *D. marginatus* and *D. reticulatus* in Europe range from Portugal to Ukraine (continue to the east of Kazakhstan), Turkey and probably to the northern parts of Iran (7-10). The first report of Dermacentor ticks in Iran was documented in 1971 by Mazlum (11). This study performed among the 30 provinces and the results defined that, Dermacentor ticks were found only in six provinces (Semnan, Khorasan, Kurdistan, Ardabil, East Azerbaijan, and Zanjan) with the highest rate of distribution in Ardabil in which ticks was found to be restricted to four species; *D. niveus*, *D. margina-*

http://jad.tums.ac.ir
Published Online: March 31, 2021

tus, D. *raskemensi*s and *D. daghestanicus* (12-17). In a study 200 adult *Dermacentor* ticks (139 *D. niveus* and 61 *D. marginatus*) were collected from a sheep babesiosis infected in the Ardabil region of Iran, *B. ovis* was detected in ticks by semi-nested PCR. Based on the results obtained *D. niveus* and *D. marginatus*, which are distributed in Ardabil region of Iran, might play a crucial role in the transmission of *Babesia* infection to domestic animals (12). To date, there are no reports of *D. reticulatus* in Iran. *Dermacentor* ticks usually occurred in the mountainous area with cold climatic conditions and high altitude (15). A cross sectional study in southeastern Iran, among tick-infested dogs, three dogs were infected with *B. gibsoni*. In this study, ticks were identified and belonged to *R. sanguineus sensu lato* and no Babesia DNA was detected. This study first record of *B. gibsoni* in dogs in Iran (18). It seems that *B. canis* and *B. gibsoni* were the major species infecting dogs and causing various clinical symptoms in Iran (19-21). Based on the morphological features of the infection, it has documented that tick species including *R. bursa*, *R. sanguineus*, *R. turanicus* and *D. marginatus* have distributed in different regions of Iran. *R. bursa* and *R. sanguineus* may play the major roles as the vector of the parasite respectively in the case of animal babesiosis in Iran (13). The babesiosis infection was detected with molecular and serological methods in dogs and other wild canine (22-27). The molecular-based techniques enable differentiation of morphologically undistinguishable *Babesia* species (28). The molecular diagnostic methods (for example PCR) are cost-effective with high sensitivity and specificity and the most reliable techniques for *Babesia* DNA detection in blood (29-31). Easy application and accessibility of databases and growing amount of annotated genomic sequences in databanks caused an improvement in the phylogenetic studies on *B. canis* (31). Limited information on the canine babesiosis has been documented, while a high number of suspicious clinical cases reported in dogs from Iran. This study provides essential and valuable data to insight into the prevalence and distribution of canine babesiosis in Iran. Thereby, regarding to raise of knowledge on this parasite, the detection and characterization of the *Babesia* species and subspecies from canine babesiosis in Meshkin shahr has a great importance through application of PCR and sequencing of 18S rRNA gene sequences.

Materials and Methods

Study area, blood and spleen samples

From July 2017 to February 2018, a total of 43 blood samples were randomly collected from shepherd dogs (*Canis familiaris*) (32 males and 11 females, 9 months to 7 years old) in Meshkin Shahr, Ardabil Province, Northwest of Iran. Blood samples were collected into 0.001M EDTA-containing tubes, and transported in ice-boxes to the laboratory of protozoology, faculty of medicine, Iran University of medical sciences. Blood samples aliquoted, smears were prepared from EDTA-sampled, blood air-dried, and stained with Giemsa. Genomic DNA was extracted from each blood samples using a DNA extraction kit (Qiagen DNA Blood Mini-Kit, Germany). All samples were identified and followed the detection process using PCR.

Molecular analysis and characterization of the isolated *Babesia* species

DNA was extracted from whole blood samples using the DNA extraction kit from blood (Qiagen, Hilden, Germany) through following manufacturer instructions as previously described (22, 31). To detect *Babesia* species, the gene fragment (~550bp) from 18S rRNA was amplified and sequenced using the primers BAB GF2 (5′-GYYTTGTAATTGGAATGA TGG-3′) and BAB GR2 (5′- CCAAAGAC TTTGA TTTCTCTC-3′). All stages were performed using the previously described PCR protocol (23). Generally, reactions were performed in a total of 25µl, including 2.5µl of 10X PCR buffer, 2.0µl of dNTP (2.5mM each), 1.25U of Taq

DNA polymerase (SinaColon Co. Iran) 1.0µl of template DNA, 1.0µl of each primer (10pmol), and 16.25µl of double distilled water (Sina Colon Co. Iran). The PCR reaction was 95 °C (3min), [95 °C (30s), 55 °C (30s), 72 °C (90s)]× 35 cycles, 72 °C (5min). In the second round of PCR 418bp of DNA fragments were generated using another pair of primers, PIRO-nest F (5′-GGATAACCGTGST AATTSTAGGGC-3′) and PIRO-nest R (5′-GTGTGTACAAAGGG CAGGGACG-3′) (4). The amplified PCR products were maintained at -20 °C until analyzed. The products were run on electrophoresis in a 1.5% agarose gel containing 0.2µg of safe stain/ml in Trisacetate-EDTA buffer at 120V for 30 min and consequently transilluminated under UV light.

Ethical approve

This study was approved by admission with the ethics procedures and guidelines of the respective national the animal ethics use committees of research issued by the council of the Iran University of Medical Sciences (IR. IUMS.REC. 27899.).

Sequences analysis

Sequences subjected to online BLAST algorithm and were compared with previously registered sequences in the GenBank database. To confirm the classification of the parasite, large fragments of the 18S rRNA gene were amplified from each sample that was positive for *Babesia*. The18S rRNA genes sequences were analyzed by standard technique using a sequencer and BioEdit software (Perkin-Elmer, USA) (32). Analysis of DNA sequences and phylogenetic relationships for *B. canis* isolates and the group of isolates from dogs were aligned using Clustal W software (33). A phylogenetic tree was created using alignments performed with neighbor joining (NJ) phylogenetic tree using Kimura-2-Parameter algorithm with bootstrap as the tree construction method (34). Furthermore, phylogenetic analysis of gene sequences were performed with maximum likelihood method with MEGA 7.0 software. The representative sequence was annotated in the GenBank database with accession number MN173220, MN173221, MN173222 and MN 173223 (Table 1). To assess *B. canis* genotypes (4, 35), the obtained sequences were compared with the members from genotype A (AY 703072) and genotype B (AY649326).

Results

In the direct microscopic diagnostic investigation of blood smear and molecular study of blood samples revealed that four dogs 9.3% (4/43) including one female and three male dogs were infected with *B. canis* (Fig 1). In the clinical examinations, all four dogs had major symptoms of babesiosis and most of the infected dogs had fever and splenomegaly. In addition, blood parameters including hemoglobin concentration, haematocrit, RBC count, and direct bilirubin had increased (Table 1). Out of the total samples subjected to PCR, four dogs was *Babesia*-positive including one female and three males. DNA was purified from all blood samples of the collected dogs and used as the PCR template, which a 550bp band was observed in the analysis. The results of sequence analysis were the same as with the other previously annotated sequences. The nucleotide sequences from canine samples were identical to each other and had shown a 99.6–100% identity with *B. canis* derived from dogs in GenBank reference sequences originated from different countries such as Turkey (KY247106 and KF499115), China (MK256974), Slovakia (DQ869307), Estonia (KT008057), Romania (HQ662634), and Croatia (AY072926). The comparison of *B. canis* nucleotide sequences obtained in this study with genotypes A and B revealed that all our isolates were classified as genotype B and the main difference was observed in positions 490 and 491. The difference of the two genotypes in the row of adenine and guanine nucleotides are in genotype B as AG and GA in genotype A (Fig. 2). The results of phylogenetic analysis revealed that the 18S

http://jad.tums.ac.ir
Published Online: March 31, 2021

rRNA gene sequences obtained in this study matched with *B. canis* and alignments showed that all *B. canis* isolates belonged in the category of genotype B (Fig. 3). In present study, the common ancestor of genotype A and B was obtained with confidence level 99%. Genetic confidence intervals can help to better understand genealogical relationships to DNA matches.

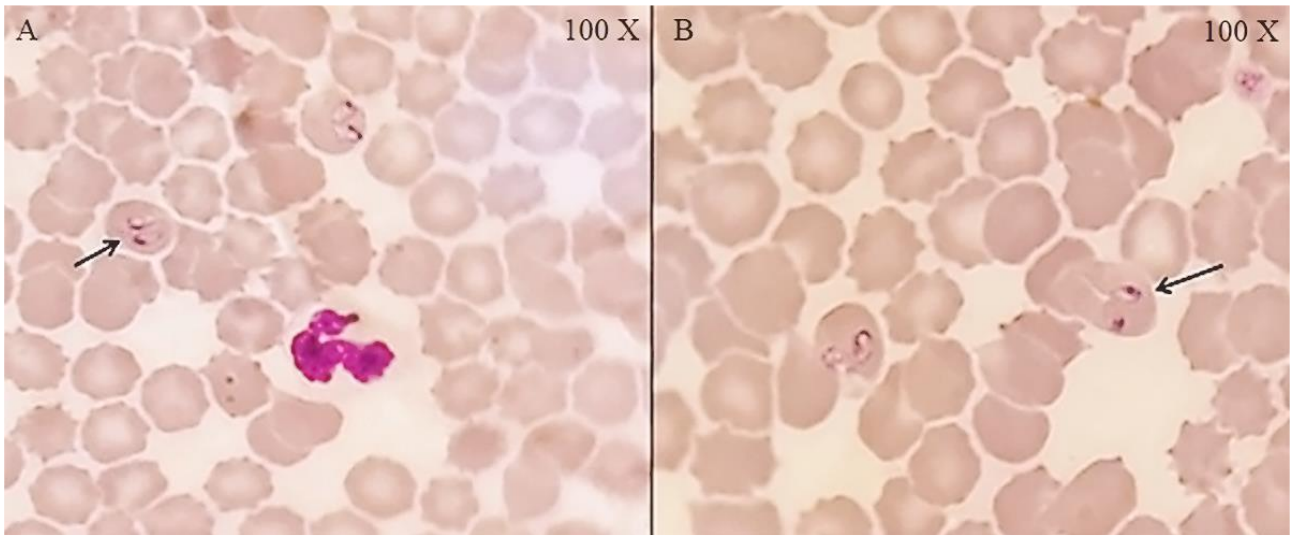

Fig. 1. Direct microscopic detection of *Babesia canis* in the blood of naturally infected dogs. Field-Giemsa stained thin smears showing various forms of *B. canis* in erythrocytes. A: Closed angle pyri form bodies of *B. canis* and B: Wide angles *B. canis* near the margin of the infected RBCs

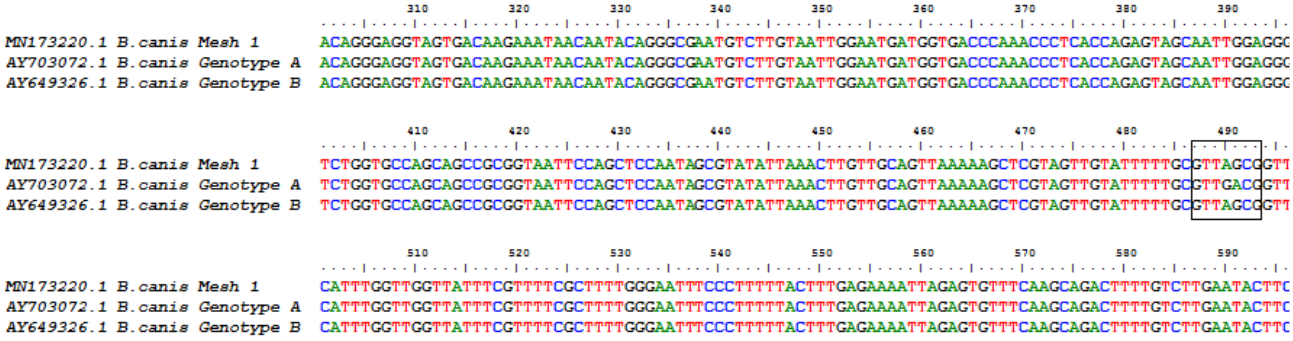

Fig. 2. Multiple sequence alignment of the partial 18S rRNA gene and the sequences of genotype A and B. In this position nucleotide changes can be seen

http://jad.tums.ac.ir
Published Online: March 31, 2021

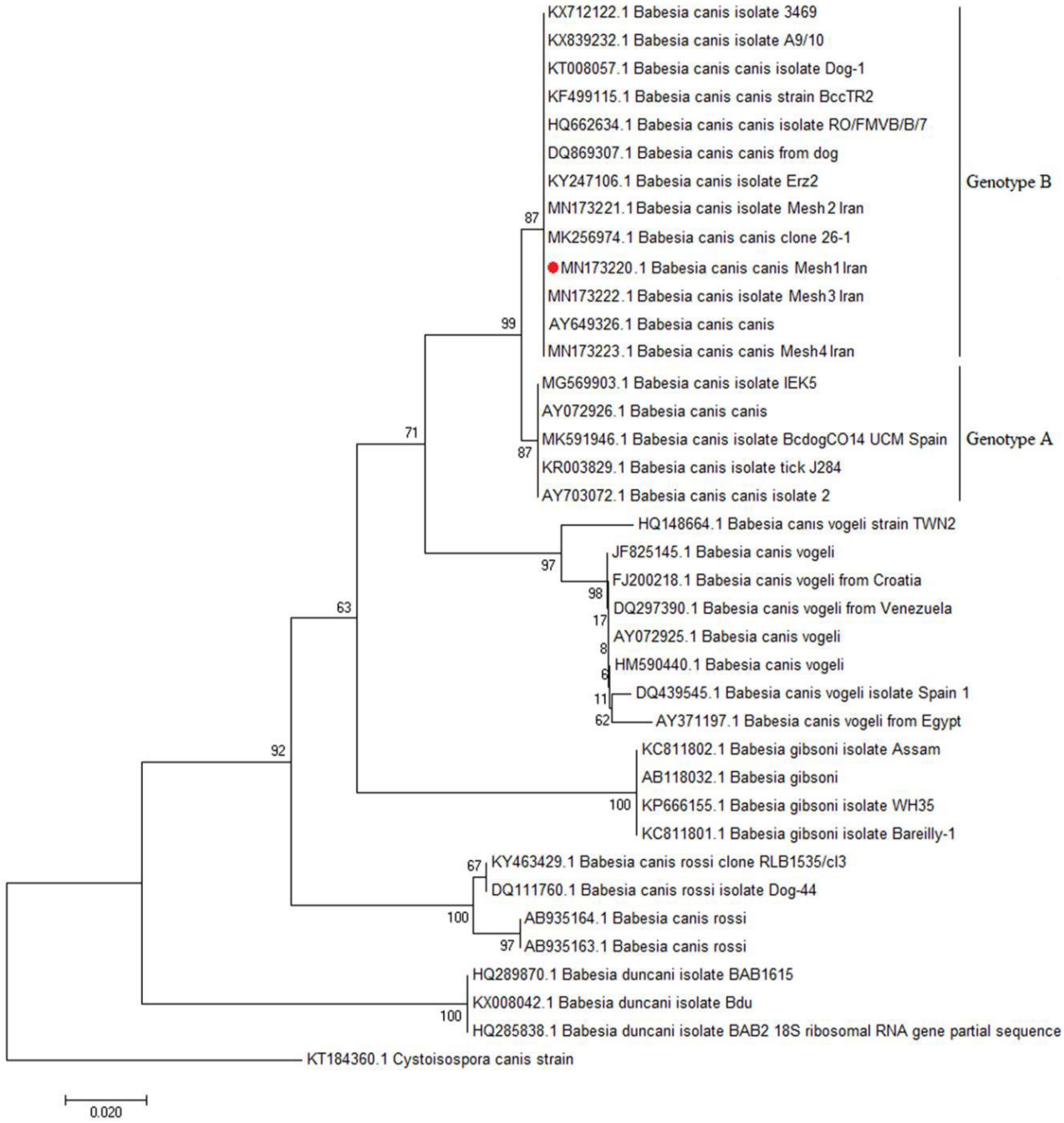

Fig. 3. Neighbor-joining analysis of canine *Babesia* sequences obtained from samples submitted. A 550bp fragment of the 18S rRNA was aligned with representative sequences derived from GenBank. Bootstrap values (1000 replications) are shown in the phylogenetic tree. Comparison of the *B. canis* sequences obtained in this study with genotypes A and B. Samples sequenced in the present study are marked with red cycle (MN173220- MN173223). The tree was inferred using the neighbor joining method of MEGA7, bootstrap values are shown at each branch point

Table 1. Principle information on the animals sampled and the *Babesia* species isolated

Samples				Pathogen	Clinical symptoms	Accession number (s)	Blood analysis*	Diagnostic investigation	Location and Coordinates
Breed	**ID of animal**	**Age**	**Sex**						
Mongrel	Mesh 1	1 year	Female	*B. canis*	Fever, splenomegaly	MN173220	RBC: 4.3 HGB: 99 HCT: 27.6% Direct Bilirubin: 1.08	PCR: positive Blood smear: positive Tissue: positive	Ag bolagh 38°20′57″N 47°39′57″E
Kurd Mastiff (Pshdar)	Mesh 2	3 years	Male	*B. canis*	Fever, vomi-tin, lethargy	MN173221	RBC: 4.8 HGB: 90 HCT: 26.1% Direct Bilirubin: 1.01	PCR: positive Blood smear: positive Tissue: positive	Parikhan 38°24′51″N 47°38′38″E
Anato-lian Karabas	Mesh 3	4 years	Male	*B. canis*	Fever, icter, splenomegaly	MN173222	RBC: 4.3 HGB: 91 HCT: 29.1% Direct Bilirubin: 1.15	PCR: positive Blood smear: Not tested Tissue: positive	Qurt tappeh 38°25′43″N 47°37′29″E
Mongrel	Mesh 4	14 months	Male	*B. canis*	Cough, splenomegaly	MN173223	RBC: 4.1 HGB: 97 HCT: 26.6% Direct Bilirubin: 1.12	PCR: positive Blood smear: positive Tissue: positive	Qara darvish 38°56′43″N 47°28′48″E

*Normal ranges: Red blood cell (RBC) count, 4.6–10×109/L; Hemoglobin concentration (HGB), 93–153g/L; Haematocrit (HCT), 28–49%; Direct bilirubin, 0.15±0.01

http://jad.tums.ac.ir
Published Online: March 31, 2021

Discussion

In this study due to the most availability of 18S rDNA sequences from *B. canis* in the GenBank, the 18S rDNA was used to search for the intraspecific variability and the most available abundant *B. canis* sequence. Out of the 43 samples subjected to PCR, 9.3% (4/43) were found to be positive for *Babesia* infection. This is the first study of a molecular detection and identification of *B. canis* infection in dogs from Iran and our results revealed that *B. canis* was prevalent in Meshkin Shahr, Iran. On the basis of 18S rRNA gene sequence analysis, genetic heterogeneity of *B. canis* has been reported in Poland, Croatia, Estonia, Lithuania, Hungary and china (4, 22, 35). Two genotypes of *B. canis*, includes A and B, have been documented so far, and have shown to have variable virulences (4, 36). The results provided principle information toward a better understanding of the epidemiology of canine babesiosis in Iran and prepared the situation for implementation of an effective control planning on babesiosis. A variable interspecies pathogenicity of the *B. canis* genotypes stated by previous studies (1, 4, 25, 28). The clinical manifestations of *B. canis* infection are mild to acute, and the severity of disease has a significant relationship with the species of *Babesia* causing infection (4). There are few reports and studies on *Babesia* spp. in Iran, while a widespread distribution of the parasite vector and suitable weather condition were observed in some areas of Iran (18, 37). Therefore, there is the probability canine babesiosis and establishment of an infection chain in some geographic areas of Iran. Niak et al. (1973) studied the blood parasites of 155 dogs and one fox (*Vulpes vulpes*) in the north of Iran, *B. canis* was just found in one splenectomized dog and *B. gibsoni* was found from fox (21). Jalali et al. (2013) applied a PCR method in the study and documented that the prevalence of canine babesiosis was 0.36% (20). In another study, Akhtardanesh et al. (2016) detected 60 tick-infested anemic dogs, among which three dogs (5%) were positive through using a genus-specific PCR and all infected with *B. gibsoni*. None of the collected ticks was positive at the *Babesia* specific PCR (18). In the collected blood samples of dogs from seven regions of Shiraz in south of Iran, only one positive sample was infected with *B. canis* (19). The results provided useful data on the distribution of *B. canis* genotypes in dogs from Iran, and showed the necessity to use a molecular-based analysis for an accurate diagnosis of canine babesiosis. The PCR-based analysis demonstrated that the molecular techniques can a highly sensitive easy to use and cost-effective tools for the simultaneous detection and differentiation of *B. canis* genotypes. However, since a limited number of target gene sequences are currently available for molecular detection of this parasite, any consideration on the population genetics of *Babesia* in the study areas would be highly scrupulously (38). Genetic intraspecific variability is a vital mechanism for piroplasm parasite survival in hosts (39). It is proven that *B. canis* transmitted by *D. reticulatus*, and the distribution area of the parasite is directly related to the presence of this tick species. Although *B. canis* has been observed in dogs in Iran (19-21, 37), But so far we have no reports of *D. reticulatus* ticks in Iran. These results demonstrate that probably *B. canis* and *D. reticulatus* have infested a dog's population, at least in the northern part of Iran. *Dermacentor reticulatus*, not occurring in the Mediterranean climatic zone, is a tick of some cool regions generally in wooded areas. This ticks has a wide spread geographical overlap with *D. marginatus*. Preferred habitats are forests and swamps zones where it can survive for long periods (8). The main clinic pathological sings in *Babesia* infections were a moderate to acute disease haemoglobinuria and a mild to very severe normochromic normocytic haemolytic anemia but, the symptoms of the disease are classified based on clinical sings and severity of the infection (40).

http://jad.tums.ac.ir
Published Online: March 31, 2021

In the present study, the main clinical signs in *Babesia* infected dogs were fever, splenomegaly, vomiting, cough. Haemoglobinuria and haemolytic anemia was not seen, which may be due to host immune system status, age and stages of infection. Based on clinical signs and mortality rates, genotype B is more virulent than genotype A (4). Considering that, all of the positive cases of Babesiosis in this study were of genotype B and all of them had typical clinical symptoms of the disease, the results of this study are consistent with those of other studies (4, 36, 40).

The babesiosis infection is detected with molecular and serological methods in domestic dogs and other wild canine in the world. The basic method of diagnosis is the observing intracellular parasite, however, this method has limitations such as false positive, co infections and non-identification of the species (41). Serological analysis is a very useful diagnostic method, but has some limitations such as cross-reactivity between different *Babesia* species and it cannot differentiate between acute infection and prior exposure with the parasites (40, 41). The molecular-based techniques enable differentiation of morphologically undistinguishable *Babesia* species and the most reliable techniques for Babesial DNA detection in blood and tissue (36, 39, 42). Ardabil Province and especially Meshkin Shahr region have cold and mountainous climate with forest and swamp conditions. The presence probability of this tick, because of the proof of *B. canis* not far-fetched. It has proven that global warming and climate change will lead to a further spread of the vectors and transmitted pathogens (42). Indeed, the climate change is a global challenge, which may explain not only the increase of density and scattering of tick vectors, but also the pattern distribution their hosts, changes in periods of activity, and variations in geographical distribution (42). The studies suggest a possible role of *Dermacentor* spp. as vectors of tick-borne pathogens that affect human and animal health (12, 19). Fast diagnostic technique is necessary for the accurate determination of *Babesia* in canine that could be carried and possibly transmitted by *Dermacentor* or other related spp. More studies are needed to increase the knowledge in the epizootiology, ecology and epidemiology of canine babesiosis in Ardabil area. Epidemio-molecular studies are necessary to provide important data for the development of new vaccines and effective therapies against canine babesiosis (43). Through a novel diagnostic strategy, our study could characterize *B. canis* infection in dogs in Meshkin Shahr, Iran. Due to the increasing numbers of piroplasm species, infected dogs may state a drastic health position threat to dog's population and prevalence of animal infectious disease in Iran.

Conclusion

Our study identified the presence of *B. canis* in dogs in Meshkin Shahr, Iran, but further studies are needed on the prevalence of *Babesia* spp. in large sample dog populations from extended areas in Iran to understand better about the epidemiology of canine babesiosis and to promote an effective control program to determine the tick species diversity in dogs in different areas of Iran. The finding of this study provides essential data for subsequently define the critical importance of the molecular studies in management and prevention of the canine babesiosis in Iran.

Acknowledgements

The authors are pleased to thank Prof Mehdi Mohebali and Dr Zabihollah Zareei (Department of Medical Parasitology and Mycology, Tehran University of Medical Sciences, Iran) and Prof Lameh Akhlaghi (Department of Medical Parasitology and Mycology, Iran University of Medical Sciences, Iran), for their technical advises and valuable assistances. The authors declare that they have no competing interests.

References

1. Solano-Gallego L, Sainz A, Roura X, Estrada-Pena A, Miro G (2016) A review of canine babesiosis: the European perspective. Parasit Vectors. 9(1): 336.
2. Uilenberg G (2006) *Babesia* a historical overview. Vet Parasitol. 138(1–2): 3–10.
3. Rene-Martellet M, Moro CV, Chene J, Bourdoiseau G, Chabanne L, Mavingui P (2015) Update on epidemiology of canine babesiosis in southern France. BMC Vet Res. 11: 223.
4. Wang J, Liu J, Yang J, Liu Z, Wang X, Li Y, Luo J, Guan G, Yin H (2019) Molecular detection and genetic diversity of *Babesia canis canis* in pet dogs in Henan Province, China. Parasitol Int. 71: 37–40.
5. Zygner W, Gorski P, Wedrychowicz H (2016) New localities of *Dermacentor reticulatus* tick (vector of *Babesia canis canis*) in central and eastern Poland. Pol J Vet Sci. 12(4): 549–555.
6. Vichova B, Miterpakova M, Iglodyova A (2014) Molecular detection of co-infections with *Anaplasma phagocytophilum* and/or *Babesia canis canis* in *Dirofilaria*-positive dogs from Slovakia. Vet Parasitol. 203(1–2): 167–172.
7. Rubel F, Brugger K, Pfeffer M, Chitimia-Dobler L, Didyk YM, Leverenz S, Hans D, Olaf K (2016) Geographical distribution of *Dermacentor marginatus* and *Dermacentor reticulatus* in Europe. Ticks Tick Borne Dis. 7(1): 224–233.
8. Rar VA, Maksimova TG, Zakharenko LP, Bolykhina SA, Dobrotvorsky AK, Morozova OV (2005) *Babesia* DNA detection in canine blood and *Dermacentor reticulatus* ticks in southwestern Siberia, Russia. Vector Borne Zoonotic Dis. 5(3): 285–287.
9. Orkun O, Karaer Z (2017) Molecular characterization of *Babesia* species in wild animals and their ticks in Turkey. Infect Genet Evol Infect. 55: 8–13.
10. Aktas M, Ozubek S, Altay K, Ipek ND, Balkaya I, Utuk AE, Erdem Utuk A, Kırbas A, Şimsek S, Dumanlı N (2015) Molecular detection of tick-borne rickettsial and protozoan pathogens in domestic dogs from Turkey. Parasit Vectors. 8: 157.
11. Mazloum Z (1971) Ticks of domestic animals in Iran: Geographical distribution, host relation and seasonal activity. Iran J Vet Med. 27: 1–32.
12. Gholamreza S, Somaieh M, Roya S, Alireza B, Ghazale A, Yasin B (2017) First detection of *Babesia ovis* in *Dermacentor* spp in Ardabil area, northwest of Iran. J Vector Borne Dis. 54(3): 277–81.
13. Esmaeilnejad B TM, Asri-Rezaei S, Dalir-Naghadeh B, Mardani K, Golabi M, Arjmand J, Kazemnia A, Jalilzadeh G (2015) Determination of prevalence and risk factors of infection with *Babesia ovis* in small ruminants from West Azerbaijan Province, Iran by polymerase chain reaction. Iran J Arthropod Borne Dis. 9(2): 246–252.
14. Nabian S, Rahbari S, Shayan P, Hadadzadeh HR (2008) Identification of tick species of dermacentor in some localities of Iran. J Vet Res. 63(3): 123–126.
15. Rahbari S, Nabian S, Shayan P (2007) Primary report on distribution of tick fauna in Iran. Parasitol Res. 101 Suppl 2: 175–177.
16. Razmi GR, Glinsharifodini M, Sarvi S (2007) Prevalence of ixodid ticks on cattle in Mazandaran Province, Iran. Korean J Parasitol. 45(4): 307–310.
17. Nabian S, Shayan P, Haddadzadeh HR (2007) Current status of tick fauna in north of Iran. Iran J Parasitol. 2(1): 12–17.
18. Akhtardanesh B, Saberi M, Nurollahifard SR, Aghazamani M (2016) Molecular detection of *Babesia* spp. in tick-infested

dogs in southeastern Iran. J Dis Glob Health. 8(2): 72–77.
19. Bigdeli M, Rafie SM, Namavari MM, Jamshidi S (2012) Report of *Theileria annulata* and *Babesia canis* infections in dogs. Comp Clin Path. 21(3): 375–377.
20. Jalali R, Mosallanejad MH, Avizeh B, Alborzi R, Hamidi Nejat AR, Taghipour H (2013) *Babesia* infection in urban and rural dogs in Ahvaz District, Southwest of Iran. Arch Razi Inst. 68(1): 37–42.
21. Niak A, Anwar M, Khatibi S (1973) Canine babesiosis in Iran. Trop Anim Health Prod. 5(3): 200–201.
22. Augustine S, Sabu L, Lakshmanan B (2017) Molecular identification of *Babesia* spp. in naturally infected dogs of Kerala, South India. J Parasit Dis. 41(2): 459–462.
23. Bajer A, Rodo A, Bednarska M, Mierzejewska E, Welc-Faleciak R (2013) *Babesia canis* and tick-borne encephalitis virus (TBEV) co-infection in a sled dog. Ann Agric Environ Med. 20(3): 426–430.
24. Beck A, Huber D, Polkinghorne A, Kurilj AG, Benko V, Mrljak V (2017) The prevalence and impact of *Babesia canis* and *Theileria* sp. in free-ranging grey wolf (*Canis lupus*) populations in Croatia. Parasite Vectors. 10(1): 168.
25. Ionita M, Mitrea IL, Pfister K, Hamel D, Buzatu CM, Silaghi C (2012) Canine babesiosis in Romania due to *Babesia canis* and *Babesia vogeli*: a molecular approach. Parasitol Res. 110(5): 1659–1664.
26. Juwaid S, Sukara R, Penezic A, Mihaljica D, Veinovic G, Kavallieratos NG, Ćirović D, Tomanović S (2019) First evidence of tick-borne protozoan pathogens, *Babesia* sp. and *Hepatozoon canis*, in red foxes (vulpes vulpes) in Serbia. Acta Vet Hung. 67(1): 70–80.
27. Sukara R, Chochlakis D, Cirovic D, Penezic A, Mihaljica D, Cakic S, Valčić M, Tselentis Y, Psaroulaki A, Tomanović S (2018) Golden jackals (*Canis aureus*) as hosts for ticks and tick-borne pathogens in Serbia. Ticks Tick Borne Dis. 9(5): 1090–1097.
28. Solano-Gallego L, Baneth G (2011) Babesiosis in dogs and cats-expanding parasitological and clinical spectra. Vet parasitol. 181(1): 48–60.
29. Baneth G, Kenny MJ, Tasker S, Anug Y, Shkap V, Levy A, Shaw SE (2004) Infection with a proposed new subspecies of *Babesia canis*, *Babesia canis* subsp. *presentii*, in domestic cats. J Clin Microbiol. 42(1): 99–105.
30. Brkljacic M, Matijatko V, Kis I, Kucer N, Forsek J, Rafaj RB, Grden D, Torti M, Mayer I, Mrljak V (2010) Molecular evidence of natural infection with *Babesia canis canis* in Croatia. Acta Vet Hung. 58(1): 39–46.
31. Lyp P, Adaszek L, Furmaga B, Winiarczyk S (2015) Identification of new 18S rRNA strains of *Babesia canis* isolated from dogs with subclinical babesiosis. Pol J Vet. 18(3): 573–577.
32. Sanchez-Villeda H, Schroeder S, Flint-Garcia S, Guill KE, Yamasaki M, McMullen MD (2008) DNA Align Editor: DNA alignment editor tool. BMC Bioinformatics. 9: 154.
33. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22(22): 4673–4680.
34. Nishimaki T, Sato K (2019) An Extension of the Kimura Two-Parameter Model to the Natural Evolutionary Process. J Mol Evol. 87(1): 60–67.
35. Adaszek L, Winiarczyk S (2008) Molecular characterization of *Babesia canis canis* isolates from naturally infected dogs in Poland. Vet Parasitol. 152(3–4): 235–241.
36. Hrazdilova K, Mysliwy I, Hildebrand J, Bun-

kowska-Gawlik K, Janaczyk B, Perec-Matysiak A, Modrýa D (2019) Paralogs vs. genotypes, variability of *Babesia canis* assessed by 18S rDNA and two mitochon drial markers. Vet Parasitol. 266: 103–110.
37. Ashrafi H, Haddadzadeh H, Shirani D, Khazraiinia P, Mostofi S (2001) Histopathologic, hematologic and clinical study on canine babesiosis. Iran J Vet Med. 56: 93–96.
38. Annoscia G, Latrofa MS, Cantacessi C, Olivieri E, Manfredi MT, Dantas-Torres F, Otranto D (2017) A new PCR assay for the detection and differentiation of *Babesia canis* and *Babesia vogeli*. Ticks Tick Borne Dis. 8(6): 862–865.
39. Milanović Z, Vekić J, Radonjić V, Ilić Božović A, Zeljković A, Janac J, Spasojević-Kalimanovska V, Buch J, Chandrashekar R, Bojić-Trbojević Ž, Hajduković L, Christopher MM, Kovačević Filipović M (2019) Association of acute *Babesia canis* infection and serum lipid, lipoprotein, and apoprotein concentrations in dogs. J Vet Intern Med. 33(4): 1686–1694.
40. Davitkov D, Vucicevic M, Stevanovic J, Krstic V, Tomanovic S, Glavinic U, Stanimirovic Z (2015) Clinical babesiosis and molecular identification of *Babesia canis* and *Babesia gibsoni* infections in dogs from Serbia. Acta Vet Hung. 63 (2): 199–208.
41. Kovacevic Filipovic MM, Beletic AD, Ilic Bozovic AV, Milanovic Z, Tyrrell P, Buch J, Breitschwerdt EB, Birkenheuer AJ, Chandrashekar R (2018) Molecular and serological prevalence of *Anaplasma phagocytophilum, A. platys, Ehrlichia canis, E. chaffeenses, E. ewingii, Borrelia burgdorferi, Babesia canis, B. gibsoni* and *B. vogeli* among clinically healthy outdoor dogs in Serbia. Vet Parasitol Reg Stud Reports. 14: 117–122.
42. Beugnet F, Chalvet-Monfray K (2013) Impact of climate change in the epidemiology of vector-borne diseases in domestic carnivores. Comp Immunol Microbiol Infect Dis. 36(6): 559–566.
43. Baneth G (2018) Antiprotozoal treatment of canine babesiosis. Vet Parasitol. 254: 58–63.